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Abstract

Objectives: The anti-Wa antibody found in systemic sclerosis patients reacts with a transfer RNA (tRNA)-associated 48-kDa protein and immunoprecipitates several tRNAs. We investigated the Wa antigen and its binding to tRNA species. **Methods:** We performed molecular cloning of the Wa antigen and made its recombinant protein. To investigate Wa antigen distribution in the cell, we performed an indirect immunofluorescence study. To determine the Wa-bound tRNA species, we performed a reverse transcription (RT)-polymerase chain reaction (PCR) using the RNAs immunoprecipitated by anti-Wa antibody as templates, and synthetic primers of mammalian tRNA sequences. To clarify the tissue expression of Wa antigen, we performed quantitative and semi-quantitative PCR of the cDNA. **Results:** We demonstrated that the Wa antigen was identical to NEFA (DNA binding/EF-hand/acidic amino acid rich region), otherwise known as nucleobindin-2. A full-length and an alternative splice variant cDNA lacking exon 11 were isolated by cloning NEFA cDNA. Anti-Wa-positive sera stained both the nucleus and cytoplasm of HEp-2 cells. RT-PCR suggested that Wa binds at least six tRNA species. In human tissues, NEFA is expressed predominantly in exocrine glands. **Conclusions:** We have demonstrated that the Wa antigen is NEFA or nucleobindin-2, which binds the specific tRNA species, and is distributed in specific human tissues.

Introduction

In connective tissue diseases, autoantibodies often recognize cellular components of both the nucleus and cytoplasm. These target autoantigens are frequently associated with either nucleic acids themselves or with proteins binding to nucleic acids. The anti-Wa antibody is a transfer RNA (tRNA)-associated antibody that was reported in 1991 [1]. It was named after a patient with systemic sclerosis (SSc) in whom the antibody was first found. It reacts with a 48-kDa protein that binds several unidentified tRNAs. The electrophoretic pattern of the tRNAs immunoprecipitated by the anti-Wa antibody is different from the patterns of other known tRNA-associated antibodies, such as those binding anti-aminoacyl tRNA synthetases.

In this study, we demonstrate that the Wa autoantigen is identical to NEFA or nucleobindin-2 [2]. NEFA (DNA binding, EF-hand, acidic amino acid-rich region) was named by Barnikol-Watanabe et al. [2]. Its molecular weight is 47.713 kDa [2, 3], which is the same as that of the Wa antigen [1]. NEFA has homology with nucleobindin [4], and is therefore also known as nucleobindin-2. NEFA has a basic amino acid-rich region and is thought to bind to DNA, although there is no direct evidence for this, nor is there any direct evidence for its binding to RNA.

Here, we confirm that NEFA is identical to the Wa antigen and that it binds several tRNAs. It may be involved in regulating the secretion of proteins, as well as being involved with calcium metabolism, and could affect protein translation systems and feeding behavior via tRNAs.

Materials and methods

Sera and antibodies

The prototype serum containing anti-Wa antibody was a gift from Dr. Hajime Yamagata, National Hospital Organization, Murayama Medical Center, Tokyo, Japan. Other anti-Wa-positive sera, as described previously [1], were also used. Permission for the use of these sera was obtained from the internal review board of each institution. All sera were stored at -20°C until use.

Rabbit anti-NEFA serum was produced by inoculating a rabbit (Japanese White) by Scrum (Tokyo, Japan) with recombinant NEFA. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was purchased from Cappel (West Chester, PA, USA).

Molecular cloning of Wa-encoded cDNA

Cloning of cDNA for the Wa antigen was performed using a lambda gt11 cDNA library constructed from human liver mRNA (Clontech, Palo Alto, CA, USA) and the anti-Wa prototype serum as the probe, as described by Young and Davis [5]. Positive clones were picked and purified repeatedly until all progeny plaques were positive. cDNA inserts isolated from positive clones were ligated into pUC118 and the sequence of the cDNA insert in the plasmid was determined. Detailed homologies of nucleotide and deduced amino acid sequences were analyzed using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast).

Affinity purification of anti-Wa antibody

Affinity-purified anti-Wa antibody reacted with recombinant proteins was obtained as previously

described [6]. Briefly, recombinant proteins from each positive clone were absorbed on a nitrocellulose (NC) filter (BA-85, Schleicher & Schuell, Dassel, Germany). The filter was incubated with the diluted anti-Wa prototype serum. After washes, antibodies were eluted from the filter-bound fusion proteins.

RNA immunoprecipitation

Ten microliters of sera containing anti-Wa antibody was mixed with 2 mg of Protein A-Sepharose CL-4B (Amersham Biosciences, Buckinghamshire, UK) in 500 µl IPP buffer (10 mM Tris-HCl, 500 mM NaCl, 0.1% Nonidet P-40, pH 8.0) and incubated at 4°C for 2 h. The Sepharose particles were then washed three times with IPP buffer and incubated with extracts from 6×10^6 sonicated HeLa cells per sample in 700 µl NET-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Nonidet P-40, pH 7.5) at 4°C for 2 h. Following five washes with the NET-2 buffer, nucleic acids were extracted from the immunoprecipitates with phenol/chloroform/isoamyl alcohol (50:50:1) and precipitated with ethanol. They were separated by 8 M urea-10% polyacrylamide gel electrophoresis and visualized by silver staining using a Bio-Rad Silver Stain kit (Bio-Rad Laboratories, Hercules, CA, USA) [7].

Immunoblotting with anti-Wa-positive sera

Cloned phages were infected into *Escherichia coli* Y1089 to develop lysogens. Fusion proteins were prepared, separated on sodium dodecylsulfate (SDS)-7.5% polyacrylamide slab gel, and transferred to an NC membrane. After blocking with 2% skim milk, patient sera were preabsorbed with *E. coli* lysates (1:100), and then goat anti-human IgG conjugated to alkaline phosphatase (1:500) (Promega, Madison, WI, USA) and NBT/BCIP (Promega) were used for immunoblotting.

Preparation of recombinant NEFA

cDNAs encoding NEFA were prepared from HeLa cell messenger RNA (mRNA) by reverse transcription (RT)-polymerase chain reaction (PCR) according to the published sequence of NEFA [2], and cloned using a TOPO-XL PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). The products were verified by sequencing. NEFA cDNA was inserted into the pET151 plasmid using a Champion pET Directional TOPO Expression Kit (Invitrogen), expressed together with a His tag and a V5 epitope tag. Recombinant NEFA was expressed in *E. coli* (BL21 Star; Invitrogen) and purified via the His tag on BD TALON Superflow Resin (BD Biosciences, Mountain View, CA, USA).

Protein immunoprecipitation

Ten microliters of sera was mixed with 2 mg Protein A-Sepharose CL-4B in 500 µl IPP buffer and incubated at 4°C for 2 h. The Sepharose particles were washed three times with IPP buffer and then incubated with sonicated *E. coli* extracts containing recombinant NEFA at 4°C for 2 h. The Sepharose particles were washed five times with IPP buffer. Immunoprecipitates were separated by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE), and recombinant NEFA was visualized by western blotting using the His Western Kit (BD Biosciences) according to the manufacturer's instructions.

Indirect immunofluorescence

HEp-2 cells on slides (FLUORO HEPANA test, MBL Laboratories, Aichi, Japan) were stained with anti-Wa-positive sera or normal human serum according to the manufacturer's protocol. When the

cells were stained with rabbit anti-NEFA sera, FITC-conjugated goat anti-rabbit whole IgG (Cappel) was used instead of the FITC-conjugated goat anti-human IgG from the kit. These slides were then examined under a fluorescence microscope (AxioVert200; Carl Zeiss, Göttingen, Germany).

Reverse transcription (RT)-PCR of tRNAs

Twenty-two sets of primers were synthesized by reference to known mammalian tRNAs. These primer sets were predesigned and made by Sigma-Genosys (Hokkaido, Japan). tRNAs immunoprecipitated by autoantibodies were used as templates, and total RNA from HeLa cells as the positive control. RT-PCR was performed using One Step RNA PCR Kits (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. PCR products were separated on 10% native polyacrylamide gels, stained with ethidium bromide, and visualized by UV light.

Quantitative real-time PCR (qPCR)

To examine the distribution of NEFA in various tissues and organs, qPCR was performed. Primer sets for wild-type and splice variant NEFA were designed by reference to the cloned NEFA cDNAs. The specificity of the primer sets was verified by PCR, using the cloned NEFA cDNA as templates. Quantitative PCR was performed-using Human MTC Panel I and II (Clontech) human tissue cDNA templates-with SYBR Premix Ex Taq (TaKaRa) on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Quantification was performed with the cloned NEFA cDNAs after normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Semi-quantitative PCR

Primer sets were designed on both sides of exon 11 (lacking in the splice variant NEFA) by reference to the cloned NEFA cDNA. Semi-quantitative PCR was performed-using Human Rapid-Scan (OriGene, Rockville, MD, USA) as human tissue cDNA templates-with La Taq (TaKaRa). The PCR products were separated on 2% agarose gels and stained with ethidium bromide.

Results

Cloning and sequencing of cDNA encoding the Wa antigen

Using the anti-Wa prototype serum as a probe, we screened 10^6 clones from the lambda gt11 human cDNA library and isolated 10 positive clones, termed Wa-1 to Wa-10. We examined whether affinity-purified antibodies from these 10 clones could immunoprecipitate tRNA. Affinity-purified antibody from clone Wa-1, as well as anti-Wa serum, immunoprecipitated tRNA, so we assumed that Wa-1 cDNA encoded the Wa antigen (Fig. 1a). The affinity-purified antibody from clone Wa-2 immunoprecipitated smeary nucleic acid components, including tRNA, so Wa-2 was also used for the further study. Using fusion proteins of Wa-1, Wa-2, and β-galactosidase (96 kD), we performed immunoblot studies with the prototype anti-Wa serum, 5 anti-Wa-positive sera, and a normal human serum. The Wa-1 fusion protein was recognized by the prototype anti-Wa serum and all five Wa-positive sera, whereas the Wa-2 fusion protein was recognized only by the prototype anti-Wa serum (Fig. 1b). These data indicated that the Wa-1 clone corresponded to the Wa antigen. The cDNA inserts obtained from recombinant clone phages were sequenced, and a homology search in NCBI

BLAST revealed that the nucleotide sequence and deduced amino acid sequence of Wa-1 insert cDNA were completely identical to those of partial human NEFA protein at nucleotide sequence positions (np) 434-1200.

Confirmation that Wa and NEFA are identical

NEFA cDNAs were cloned from HeLa cell mRNA and used to produce recombinant NEFA protein. We obtained two types of cDNAs from this cloning, one of which encoded the target-length NEFA and the other, which encoded a 90-bp-shorter variant. A homology search with the 90-bp-deleted sequence in NCBI BLAST identified a sequence on human chromosome 11. We compared NEFA cDNA and chromosome 11, and found that NEFA cDNA consists of 14 exons (Fig. 1c), in agreement with a previous report [2]. The shorter splice variant lacked exon 11 (np 1132-1221), which encodes one of the two EF-hands. We constructed a recombinant wild-type NEFA (rNEFAw) with a His tag and a V5 epitope tag, using a Champion pET Directional TOPO Expression Kit (Invitrogen). By direct western blotting, rNEFAw after SDS-PAGE separation was found not to react with anti-Wa sera (data not shown). Therefore, we carried out anti-Wa sera immunoprecipitations of sonicated *E. coli* extracts expressing rNEFAw (Fig. 2a). All six anti-Wa-containing sera reacted with rNEFAw but not with anti-Jo-1 serum or normal human serum.

We produced hyperimmune anti-NEFA sera by inoculating two rabbits with purified rNEFAw. Using these hyperimmune anti-NEFA sera, the reactivity between NEFA and tRNA was verified by RNA-immunoprecipitation of HeLa cell extracts. The serum of one rabbit immunized with rNEFAw immunoprecipitated several tRNAs that were bound more weakly but appeared to be identical to the tRNAs recognized by anti-Wa sera (Fig. 2b).

To investigate the distribution of Wa antigen and NEFA in the cell, an indirect immunofluorescence study was performed. We used sera apparently containing only anti-Wa antibody. We did not detect other known autoantibodies in these sera when testing by RNA and protein immunoprecipitation assays. Anti-Wa-positive sera stained both the nucleus and cytoplasm of HEp-2 cells (Fig. 2c). Anti-NEFA rabbit serum showed a similar staining pattern at the high dilution of 1:320. A lower dilution of 1:5 showed a characteristic perinuclear staining similar to that in the Golgi complex, which is consistent with the distribution of NEFA according to a previous report [8, 9]. One of the four anti-Wa sera also showed perinuclear staining similar to that in the Golgi complex.

tRNA species associated with Wa/NEFA

To determine the species of Wa-bound tRNAs, RT-PCR was performed using the RNAs immunoprecipitated with the anti-Wa or anti-NEFA antibody as templates, and synthetic primers of 22 known mammalian tRNA sequences (Fig. 3a). We searched for human tRNA sequences in PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and found 15 of 20 species of tRNAs at that time. Five human tRNA sequences (for cysteine, aspartic acid, glutamic acid, isoleucine, and tryptophan) could not be found and were therefore replaced by other mammalian tRNAs (rat, mouse, or bovine). Because each set of tRNA has many isotypes, one of each tRNA species was used arbitrarily. Glycine and arginine tRNAs had diverse sequences, and we selected two sequences for each tRNA species. All

tRNA species were successfully amplified by RT-PCR using HeLa cell total RNAs (Fig. 3bA), and the PCR products were verified by sequencing. RT-PCR products derived from RNA immunoprecipitated by anti-EJ (anti-glycyl-tRNA synthetase) showed two glycine tRNA bands, as expected (Fig. 3bB). With anti-Wa immunoprecipitated tRNAs, many species were amplified. Of these, tRNAs for arginine, glycine, histidine, methionine, lysine, and proline were easily detected even when the number of PCR cycles was reduced, strongly suggesting that these were the Wa-bound tRNAs (Fig. 3bC). The same species were amplified with anti-NEFA-immunoprecipitated tRNAs (Fig. 3bD).

Tissue distribution of NEFA

To establish the tissue expression of wild-type NEFA (NEFAw) and splice variant NEFA (NEFAsv), we performed quantitative and semi-quantitative PCR using human tissue cDNA panels. For qPCR of NEFA, 3' primers were designed on the splice site of exon 11 in order to distinguish between NEFAw and NEFAsv (Fig. 4a). A primer set for NEFAw amplified its template cDNA, but not NEFAsv cDNA, and vice versa (data not shown). Human MTC Panel I and II (Clontech) were used as tissue cDNA templates and normalized against GAPDH cDNA (Fig. 4b). NEFA cDNA was detected in many tissues, with particularly strong expression observed in the pancreas, placenta, and testis. NEFAsv was expressed at a much lower level than the wild-type, but with a similar tissue distribution. For the semi-quantitative PCR of NEFA, primers were designed on both sides of exon 11, and these were thus able to distinguish between NEFAw cDNAs (260 bp) and NEFAsv cDNAs (170 bp) (Fig. 4a). Human Rapid-Scan (OriGene) was used as a source of tissue cDNA templates, normalized against β-actin cDNA and tested at four concentrations (×1000, ×100, ×10, ×1). Semi-quantitative PCR showed a distribution of NEFAw and NEFAsv similar to that shown by qPCR, particularly in the pancreas, placenta, and testis, and also in the salivary gland (Fig. 4c).

Discussion

The anti-Wa antibody was first reported in 1991 [1]. It was named after the SSc patient in whom the antibody was discovered. In the first report, the anti-Wa antibody was detected in the sera of 4 of 130 SSc patients, but it was not detected in the sera of patients with other systemic autoimmune diseases or in the sera of healthy controls. In addition to its presence in some SSc patients, it was also reported in one Sjögren's syndrome (SS) patient [10] and two polymyositis/dermatomyositis (PM/DM) patients [11]. The electrophoretic pattern of the tRNAs immunoprecipitated by anti-Wa antibody is different from that of other known tRNA-associated antibodies, such as those binding anti-aminoacyl tRNA synthetases in PM/DM. The anti-Wa antibody has been reported mainly in SSc, and the seven reported patients were all females, and out of them, in whom Raynaud's phenomenon, interstitial lung disease, and cardiomegaly were found in 6, 4, and 3 patients, respectively. Two patients had SS as an associated disease and one of these two had anti-SS-A/Ro and anti-SS-B/La antibodies. The other patient had anti-Ki and anti-RNA polymerase antibodies [1, 12, 13]. The two myositic patients with the anti-Wa antibody were a male and a female and both showed shrinking interstitial lung disease, while the female patient showed mechanic's hand [11], these being clinical

features associated with anti-aminoacyl tRNA synthetase antibodies in PM/DM [14, 15].

In the present study, we have demonstrated that the Wa autoantigen is identical to NEFA, otherwise known as nucleobindin-2. NEFA has a basic amino acid-rich region and is thought to bind to DNA via this domain [2]. We confirmed that NEFA is identical to the Wa antigen and that it binds several tRNAs, including those for arginine, glycine, histidine, methionine, lysine, and proline. The intracellular localization of NEFA differs in the different cell lines investigated. NEFA has been reported to be located in the cytomembrane and cytoplasm of the KM3 lymphoblastic leukemia cell line [2], the Golgi apparatus of HeLa cells [8, 9], and the endoplasmic reticulum and nuclear membrane of the mouse embryonal carcinoma line P19 [16]. We have shown in the present study that Wa/NEFA is expressed in both the nucleus and cytoplasm, in agreement with previous reports.

The NEFA gene is located on chromosome 11 and consists of 14 exons [17, 18]. It was detected in a study of the locus responsible for Usher syndrome type 1c (located at 11p14-15.1), but no NEFA mutation could be found in these patients. The cloning of NEFA cDNA from HeLa cells enabled us to obtain a previously reported full-length cDNA and a novel 90-bp-shorter variant. The latter variant lacked exon 11, which encodes one of the two EF-hands at the C-terminal end. We could not demonstrate the expression of the variant NEFA protein or its binding ability with tRNA, but we found that its mRNA, like that of the wild-type full-length cDNA, was expressed in many tissues, albeit at a much lower level than the wild-type full-length cDNA. The variant NEFA may have binding ability with tRNA via the basic amino acid- rich region, but we could not demonstrate the binding ability of wild-type NEFA or the splice variant NEFA with recombinant proteins (data not shown).

NEFA can bind two Ca^{2+} ions by means of the two EF-hands, but mutated NEFA with the EF-hand of the N-terminal side artificially deleted no longer binds Ca^{2+} [16, 19, 20]. Necdin (neurally differentiated embryonal carcinoma-derived protein) [16], TNFR1 (tumor necrosis factor receptor 1), and ARTS-1 (aminopeptidase regulator of TNFR1 shedding) [21] have been reported to bind NEFA. Necdin, which was discovered in murine embryonal carcinoma P19 cells that were caused to differentiate into postmitotic neurons, is also expressed in normal postmitotic neurons [22]. Necdin interacts with NEFA via two EF-hands [16]. Caffeine-evoked cytosolic Ca^{2+} levels are increased in cells over-expressing NEFA, and this phenomenon is enhanced by the co-expression of Necdin [16]. Exocrine stimulation increases cytoplasmic Ca^{2+} concentration in exocrine cells.

NEFA binds to ARTS-1 and TNFR1 in a Ca^{2+}-dependent manner [21]. TNFR1 is released via two different mechanisms: (1) proteolytic cleavage and shedding of the ectodomain from the cell surface; and (2) the release of full-length TNFR1 within exosome-like vesicles. The NEFA fragment, which contains the acidic region and part of the EF-hand of the C-terminal side, interacts with the ARTS-1 protein. Both NEFA and ARTS-1 co-immunoprecipitate primarily with TNFR1, and the formation of this tripartite complex depends on Ca^{2+}. The extracellular release of TNFR1 is reduced when NEFA is knocked down, and the release is also reduced by Ca^{2+} chelation.

NEFA is predicted to be cleaved into three fragments by prohormone convertases [23, 24]. The

intracerebroventricular injection of NEFA or its N-terminal 82-amino-acid fragment lacking the signal peptide caused reduced feeding behavior in rats. This fragment was named nesfatin-1 (NEFA/nucleobindin 2-encoded satiety- and fat-influencing protein). Other fragments generated by prohormone convertases, nesfatin-2 and nesfatin-3, as well as a NEFA mutant that could not be cleaved by prohormone convertases, did not decrease food intake. On the other hand, NEFA is cleaved by caspases at sites different from the prohormone convertase sites during apoptosis [24]. While nesfatin-1 was initially identified in hypothalamic nuclei, NEFA and nesfatin-1 are also expressed in peripheral tissues such as adipose tissue, gastric glands, the submucosal layer of the duodenum, pancreatic β-cells, and reproductive organs [25]. Nesfatin-1 enhances glucose-induced insulin secretion by promoting Ca^{2+} influx in mouse islet β-cells [26]. In our study, NEFA was associated with several tRNAs, including those for arginine, glycine, histidine, methionine, lysine, and proline, and possibly others. Interestingly, histidine, methionine, and lysine are essential amino acids that cannot be synthesized in the human body, while arginine, glycine, and proline are conditionally essential [27]. Thus, NEFA might monitor nutritional conditions by binding to aminoacyl or non-aminoacyl tRNAs and thereby influence the feeding behavior and immune responses mediated by nesfatin-1 or TNFR1, respectively.

In summary, we have identified the species of tRNA binding with NEFA, as well as the NEFA splice variant lacking exon 11, and its distribution in human tissues. The physiological role of NEFA remains unclear, but, together with Ca^{2+}, it may be involved in secretory systems and could affect protein translation and feeding behavior via tRNAs.

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Conflict of interest

None.

Abbreviations

SSc, Systemic sclerosis; rNEFAw, Recombinant wild-type NEFA; NEFAw, Wild-type NEFA; NEFAsv, Splice variant NEFA; SS, Sjögren's syndrome; PM/DM, Polymyositis/dermatomyositis.

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FIGURE LEGENDS

Figure 1

(a) Anti-Wa serum was affinity purified with recombinant proteins Wa-1 to Wa-10. RNA immunoprecipitation was then performed with each affinity-purified anti-Wa serum (*lanes 1-10*). Lane T: electrophoresis of total RNA. Lane C: no RNAs immunoprecipitated by normal human serum. (b) β-Galactosidase (lane 1), Wa-1 fusion protein (lane 2) and Wa-2 fusion protein (lane 3) were electroporated and immunoblotted with anti-Wa positive-sera (lane a-f) and normal human serum

(lane g). (c) NEFA (DNA binding/EF-hand/acidic amino acid rich region) cDNAs. Full-length (wild: w) cDNA and splice-variant (sv) cDNA lacking exon 11 were cloned from HeLa cell total cDNAs. Exons of NEFAw cDNA are shown as nucleotide sequence positions (n.p.). Specific regions of the NEFA protein are shown as amino acid sequence positions (a.p.).

Figure 2

(a) Immunoprecipitation of recombinant wild-type NEFA (rNEFAw) by anti-Wa antibody. rNEFAw was immunoprecipitated by sera and western-blotted by anti-His antibody. Anti-Wa sera (lanes 1-6), anti-Jo-1 serum (lane 7) and normal human serum (lane 8) are shown. (b) tRNAs immunoprecipitated by anti-NEFA antibody. Serum from a rabbit immunized with recombinant NEFA (lane R) or anti-Wa antibody (lane Wa) was used to immunoprecipitate several tRNAs. Anti-Wa serum in this assay contained anti-SS-A/Ro antibody and immunoprecipitated Y1-5 RNAs. Lane T: electrophoresis of total RNA. (c) (A to D) HEp-2 cells stained with four different anti-Wa-positive sera not containing any other antibodies. (E and F) HEp-2 cells stained with anti-NEFA antibody (Ab) diluted 1:320 or 1:5. (G) HEp-2 cells stained with normal human serum.

Figure 3

(a) Transfer RNA sequences and primers. Anticodons are shown in bold characters. Primer positions are underlined. [a] Mouse tRNA. [b] Rat tRNA. [c] Bovine tRNA. (b) Reverse transcription-polymerase chain reaction (RT-PCR) performed with 22 sets of mammalian tRNA primers and HeLa cell total RNAs (A) or tRNAs immunoprecipitated by antibodies (B, C, D). (A) RT-PCR with HeLa cell total RNAs as template. (B) RT-PCR with tRNAs immunoprecipitated by anti-EJ (glycyl-tRNA synthetase) sera. (C) RT-PCR with anti-Wa-immunoprecipitated tRNAs. (D) RT-PCR with anti-NEFA-immunoprecipitated tRNAs.

Figure 4

(a) Positions of NEFA primers for quantitative and semi-quantitative PCR. A: Primer sequences for quantitative PCR. 3' primers were designed for splice site (exon 11) in order to distinguish between NEFAw and splice variant NEFA (NEFAsv). The primer set for NEFAw amplified its template and not the NEFAsv cDNA template, and vice versa (data not shown). B: Primer sequences for semi-quantitative PCR. Primers were designed on both sides of exon 11, thus detecting both wild-type NEFA cDNAs (260 bp) and splice-variant NEFA cDNAs (170 bp). (b) Quantitative PCR of NEFA. Human MTC Panel I and II (Clontech) were used as templates and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. (c) Semi-quantitative PCR of NEFA. Human Rapid-Scan (OriGene) was used as a template, was normalized against β-actin cDNA, and used at four concentrations (×1000, ×100, ×10, ×1).

Figure 1

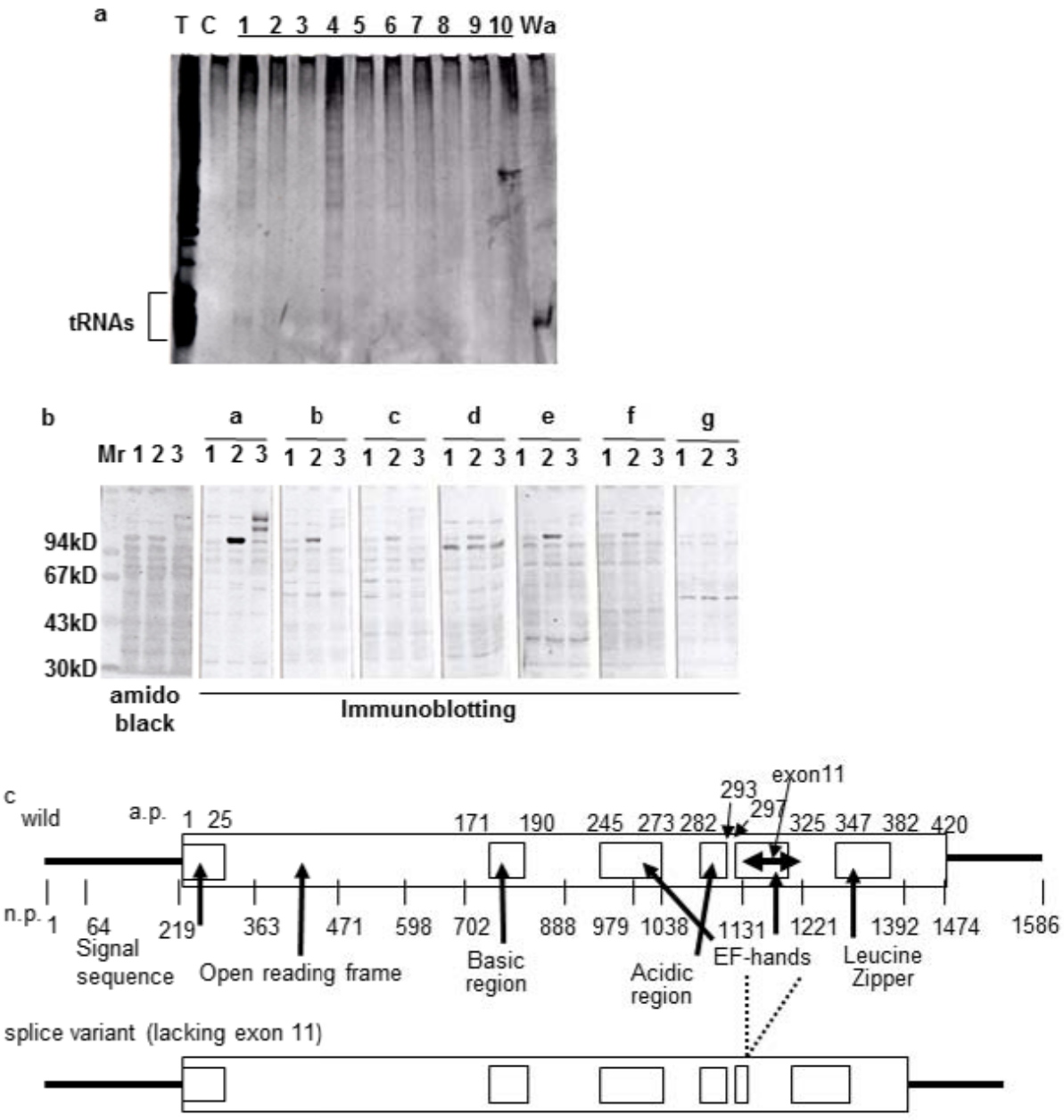


Figure 2

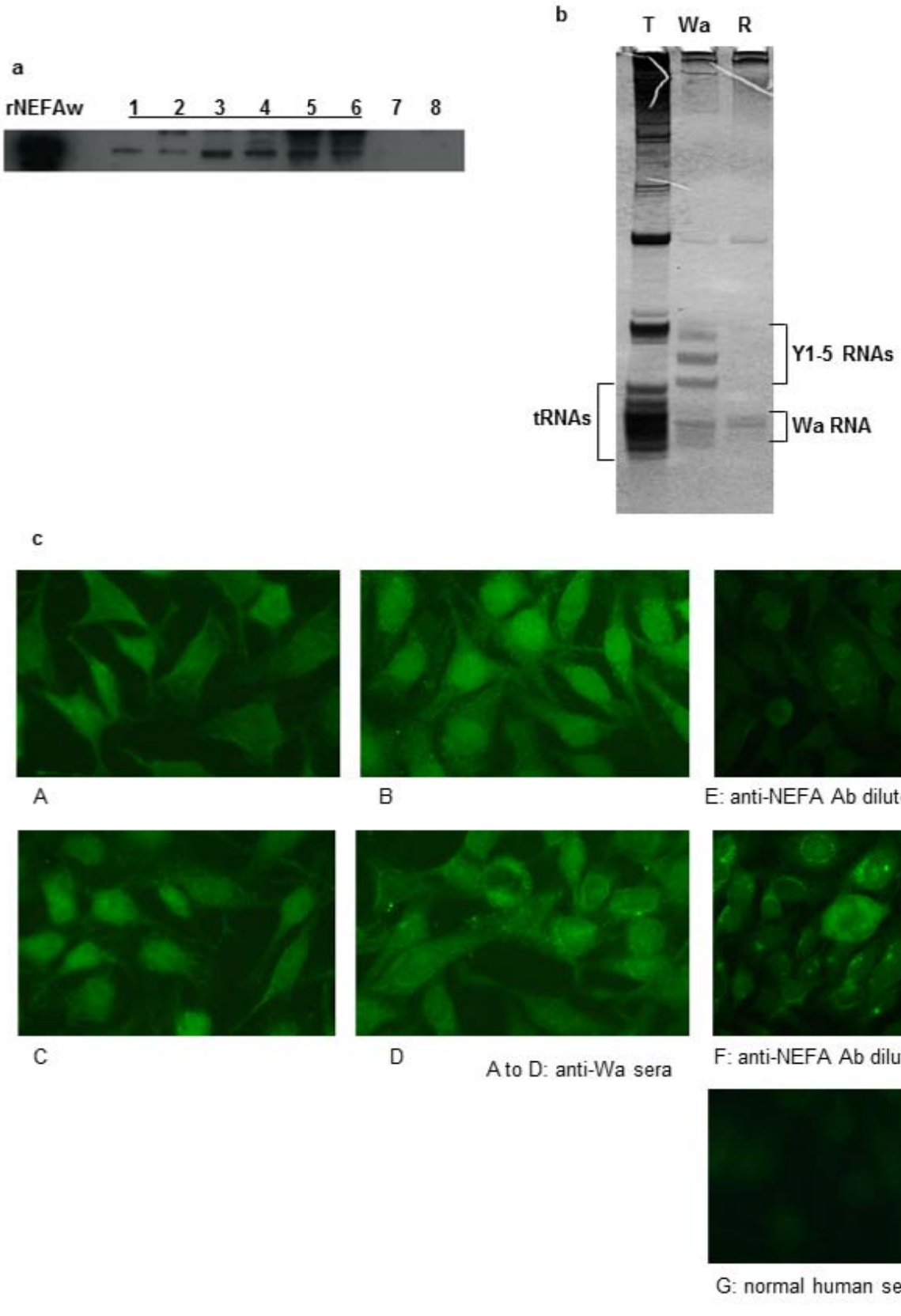


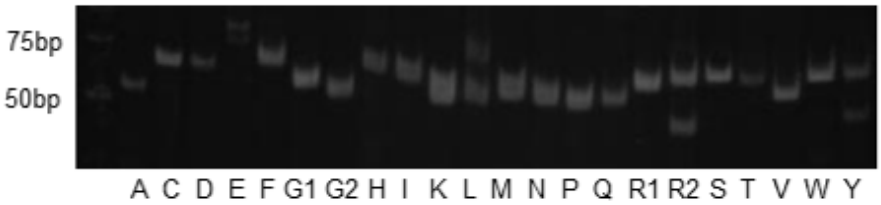
Figure 3

a

amino acid	transfer RNA sequences
A (Ala)	gggggattag ctcaaatggt agagcgctcg ctttgcttgc gagaggtagc gggatcgatg cccgca tcctccacca
C (Cys) [a]	gggggtatag ctcaggggta gagcatttga ctgcagatca agaggtccct ggttcaaatc caggt gcccctcca
D (Asp) [b]	tcctcgttag tatagtggtg agtatccccg ctcgtcacgc gggagaccgg ggttcgattc cccga cggggagcca
E (Glu) [b]	tcccacatgg tctagcggtt aggattcctg gttttcaccc aggcggcccg ggttcgactc ccggt gtgggaacca
F (Phe)	gccgaaatag ctcagttggg agagcgttag actgaagatc taaaggtccc tggttcgatc ccgggt ttcggcacca
G1 (Gly)	gcattggtgg ttcagtggta gaattctcgc ctgccacgcg ggaggcccgg gttcgattcc cggc caatgcacca
G2 (Gly)	gcgccgctgg tgtagtggta tcatgcaaga ttcccattct tgcgacccgg gttcgattcc cggg cggcgcacca
H (His)	ggccgtgatc gtatagtggt tagtactctg cgttgtggcc gcagcaacct cggttcgtat ccgagt cacggcacca
I (Ile) [a]	ggccggttag ctcagttggt tagagcgtgg tgctaataac gccaaggtcg cgggttcgat ccccgta cgggccacca
K (Lys)	gcccggctag ctcagtcggt agagcatgag actcttaatc tcagggtcgt gggttcgagc cccacg ttgggcgcca
L (Leu)	ggtagcgtgg ccgagcggtc taaggcgctg gatttaggct ccagtctctt cggaggcgtg ggttcgaatc ccacc gctgccacca
M (iMet)	agcagagtgg cgcagcggaa gcgtgctggg cccataaccc agaggtcgat ggatcgaaac catcctctgctacca
N (Asn)	gtctctgtgg cgcaatcggt tagcgcgttc ggctgttaac cgaaaggttg gtggttcgag cccaccc agggacgcca
P (Pro)	ggctcgttgg tctaggggta tgattctcgc ttggggtgcg agaggtcccg ggttcaaatc ccgga cgagccccca
Q (Gln)	ggccccatgg tgtaatggtt agcactctgg actntgaatc cagcgatccg agttcaaatc tcggt gggacctcca
R1 (Arg)	gggccagtgg cgcaatggat aacgcgtctg actacggatc agaagattcc aggttcgact cctggc tggctcgcca
R2 (Arg)	ggaccacgtg gcctaatgga taaggcgtct gacttcggat cagaagattg agggttcgaa tccctt cgtggttcca
S (Ser)	gtagtcgtgg ccgagtggtt aaggcgatgg acttgaaatc cattggggtt tccccgcgca ggttcgaatc ctgcc gactacgcca
T (Thr)	ggctccatag ctcaggggtt agagcactgg tcttgtaaac caggggtcgc gagttcaaat ctcgctgggg cctcca
V (Val)	gtttccgtag tgtagtggtt atcacgttcg cctgacacgc gaaaggtccc cggttcgaaa ccgggc ggaaacacca
W (Trp) [c]	gacctcgtgg cgcaatggta gcgcgtctga ctccagatca gaaggttgcg tgttcgaatc acgtc ggggtcacca
Y (Tyr)	ccttcgatag ctcagctggt agagcggagg actgtagatc cttaggtcgc tggttcgatt ccggct cgaaggacca

b

A. Total tRNAs



B. EJ-tRNAs

C. Wa-tRNAs

D. NEFA-tRNAs

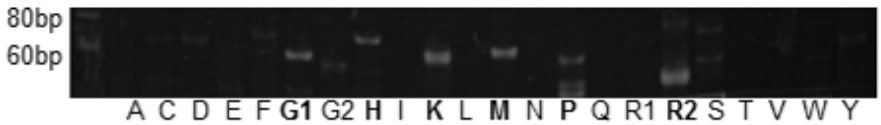
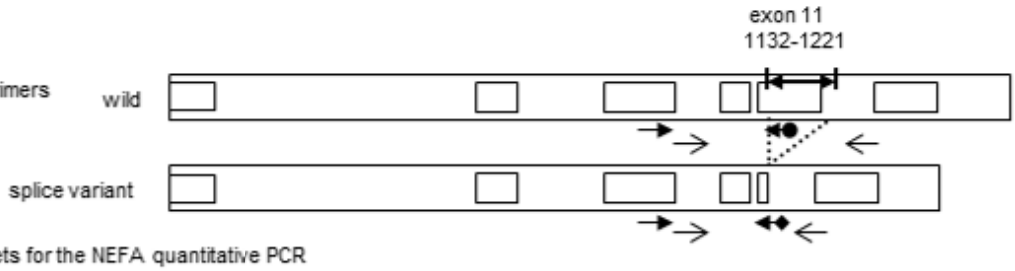


Figure 4

a. NEFA primers

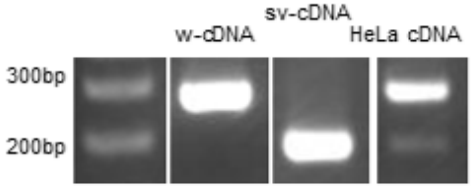


A. Primer sets for the NEFA quantitative PCR

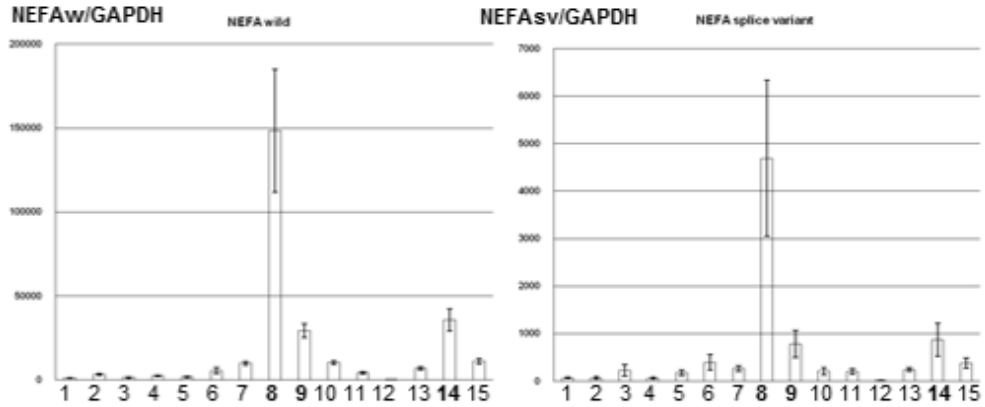
[for wild type]	sense	np975-995	→	acatgatgtcaatagtgatg
	antisense	np1160-1131(w)	◄●	gtcaccaatctgtctttgttagtatcaacc
[for splice variant]	sense	np975-995	→	acatgatgtcaatagtgatg
	antisense	np1144-1125(sv)	◄◆	gctgatctaatgtctcattc

B. Primer set for the NEFA semi-quantitative PCR

sense	np999-1024	→	cctggatgaacaagaattagaagccc
antisense	np1258-1234(w)	←	gttcttcctctgtgaagaactgttg

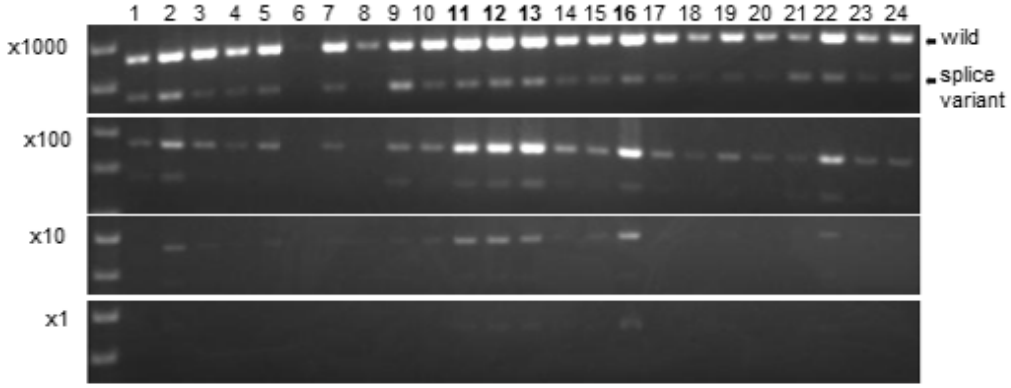


b. Quantitative PCR of NEFA



1: brain, 2: colon, 3: heart, 4: kidney, 5: leukocyte, 6: liver, 7: lung, 8: pancreas, 9: placenta, 10: prostate, 11: small Intestine, 12: skeletal muscle, 13: spleen, 14: testis, 15: thymus

c. Semi-quantitative PCR of NEFA



1: brain, 2: heart, 3: kidney, 4: spleen, 5: liver, 6: colon, 7: lung, 8: small Intestine, 9: muscle, 10: stomach, 11: testis, 12: placenta, 13: salivary, 14: thyroid, 15: adrenal gland, 16: pancreas, 17: ovary, 18: uterus, 19: prostate, 20: skin, 21: plasma blood leukocytes, 22: bone marrow, 23: fetal brain, 24: fetal liver